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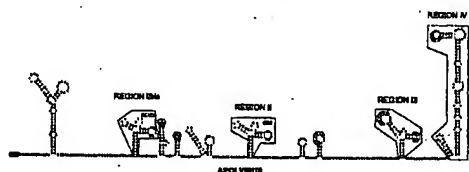
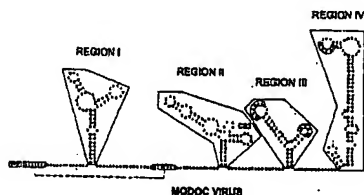
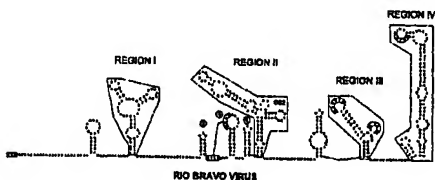
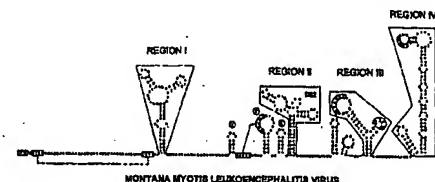
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(54) Title: FLAVIVIRUS REPLICATION



(57) Abstract: The present invention relates to the modification of the 3'UTR of a vector-borne flavivirus, more particularly the modification of one or more nucleotides within the 3'LSH of the 3'UTR, more particularly modifying one or more nucleotides within pentanucleotide sequence 5'-CAC(A/C)G-3' in the 3' UTR of such a virus in order to reduce the replication of a virus in a vector or vector cell. The present invention also relates to a modified nucleotide sequences and modified viruses comprising a functional 3'UTR of a flavivirus and characterized by one or more modified nucleotides within the 3'LSH of the 3'UTR, more particularly by one or more modified nucleotides within the CAC(A/C)G pentanucleotide motif in the 3' UTR sequence. This invention is applicable for the creation of a vaccine where there is no risk for transmitting the virus particles from one organism to another through a vector.



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## FLAVIVIRUS REPLICATION

## FIELD OF THE INVENTION

The present invention relates to a method for inhibiting the replication of viruses and to a method for inhibiting the transmission of viruses from one organism to another, such as from a vector to its host organism (human, animal) and vice versa. This method can be applied in vaccination strategies to inhibit the spreading of a virus from a vaccinated organism (human, animal) to another through a vector (e.g. mosquito or tick). The present invention also relates to nucleotide sequences, vaccines using the nucleotide sequences, modified virus and pharmaceutical compositions comprising the modified virus.

## BACKGROUND OF THE INVENTION

RNA viruses are divided in several families including the Arenaviridae, the Picornaviridae, Retroviridae, Coronaviridae, Mosaic Viruses, Orthomyxoviridae and the Flaviviridae among others.

The family of the Flaviviridae consists of nearly 80 viruses and can be subdivided into three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. The *Flavivirus* genus contains several human pathogens including yellow fever virus (YFV), dengue virus (DENV), and Japanese encephalitis virus (JEV). The *Pestivirus* genus is home to bovine viral diarrhea, classical swine fever virus and border disease virus of sheep. The genus *Hepacivirus* consists, among others, of hepatitis C virus. Hepatitis G virus (also known as GB virus C) is classified within the family of the *Flaviviridae* but has not (yet) been assigned to a genus.

Members of the *Flavivirus* genus are, based on their vector, classified into three groups: (i) flaviviruses that are transmitted by mosquitoes (mosquito-borne), (ii) flaviviruses that are transmitted by ticks (tick-borne) (iii) viruses with no known vector (NKV) (Chambers *et al.*, 1990a, *Annu. Rev. Microbiol.* 44, 649-688).

All flaviviruses of human importance belong to the arthropod-borne (mosquito- and tick-borne) group and cause a variety of diseases including encephalitis and (hemorrhagic) fevers. One of the most important flaviviruses causing human disease is dengue virus (DENV). It is estimated that there are worldwide annually as many as 50 to 100 million cases of dengue fever and several hundred

thousand cases of dengue hemorrhagic fever (DHF), the latter with an overall case fatality rate of ~5% (Gubler, 1997, D.J. Gubler and G. Kuno (ed.), *Dengue and dengue hemorrhagic fever*, CAB International, Wallingford, United Kingdom, 1-23; Monath, 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91, 2395-2400). In the book "Vaccines  
5 for the 21<sup>st</sup> Century", National Academy of Sciences, 2000, it is stated that dengue hemorrhagic fever does not figure as important for the U.S. public health burden although it goes on to say that analysis of international disease burden would likely lead to results that would be more favourable.

Despite the availability of a very efficacious vaccine, yellow fever (YF) is still  
10 a major public health problem. Other important viruses of the genus include Japanese encephalitis (JE), a mosquito-borne arboviral infection and the leading cause of viral encephalitis in Asia (Hennessy *et al.*, 1996, *Lancet* 347, 1583-1586). Tick-borne encephalitis virus (TBEV) is believed to cause annually at least 11,000 human cases of encephalitis in Russia and about 3000 cases in the rest of Europe. Related viruses  
15 within the same group, Louping ill virus (LIV), Langat virus (LGTV) and Powassan virus (POWV), also cause human encephalitis. Three other viruses within the same group, Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV) and Alkhurma virus (ALKV) tend to cause fatal hemorrhagic fevers rather than encephalitis (Gritsun *et al.*, 2003, *Antiviral Res.* 57, 129-146). Other viruses are  
20 Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV) and West Nile virus (WNV). In 1999, the WNV disease appeared for the first time in the northeastern United States and has continued to date to spread across the United States and Canada.

The NKV-group holds a few viruses which have been isolated from mice or  
25 bats and for which no arthropod-borne or natural route of transmission has (yet) been demonstrated (Kuno *et al.*, 1998, *J. Virol.* 72, 73-83). To the NKV viruses belong the Modoc virus, Rio Bravo virus, Apoi virus and Montana *Myotis* leukoencephalitis virus (Charlier *et al.*, *J. Gen. Virol.* 83, 1887-1896 and *J. Gen. Virol.* 83, 1875-1885), among others.

30 No information is available about the factors that determine whether a flavivirus is able or not to infect a vector (or vector cells) like mosquitoes or ticks. Differences between the viruses can be found in virulence, replication and transmission efficiency, severeness of pathology and other properties.

The genome of the *Flavivirus* genus (Fig. 1) consists of a linear, positive-sense, single-stranded RNA molecule of about 11 kilobases (kb) in length. This RNA contains a methylated nucleotide cap (type I: m<sup>7</sup>G5'ppp5'A) at the 5' end and lacks a 3' polyadenylate tail. Surrounding the single open reading frame (ORF) are 5' and 3' untranslated regions (UTRs) of about 100 nucleotides and 400 to 700 nucleotides, respectively. These regions contain conserved sequences and predicted RNA structures that are likely to serve as *cis*-acting elements in the processes of genome amplification, translation or packaging. Translation of the genome results in synthesis of several structural (like membrane and envelope proteins) and non-structural proteins (like a protease and a polymerase).

In recent years, the genomic sequences of an increasing number of flaviviruses have been determined. The flaviviruses of which the genome sequence is available, share several overall characteristics, such as the organization of the genome, the presence of homologous protease cleavage sites and conserved motifs in those genes that are believed to be interesting antiviral targets (helicase, polymerase). Also differences between the three flavivirus groups have been described. For example, the folding of the 3' UTR of flaviviruses has revealed structural elements that are preserved (i) among members of the arthropod-borne group, (ii) between members of the NKV-group and the tick- or mosquito-borne group and (iii) in all three groups, as well as structural elements that distinguish each group from another. The pentanucleotide motif CACAG located approximately 45-61 nucleotides from the 3' terminus, in the 3' LSH of the 3' UTR of the flavivirus genome, has been put forward to designate a virus as either an NKV or a vector-borne flavivirus. This motif is predicted to be located on a side-loop of a conserved 3'-terminal secondary structure, which plays a role in the formation of a circular RNA molecule (Chambers *et al.*, 1990b; Khromykh *et al.*, 2001, *J. Virol.* 75, 6719-6728). It was found that the second position of this pentanucleotide in the 3' UTR of NKV flaviviruses (MMLV, MODV, RBV and APOIV) was either U or C, instead of A in the vector-borne flaviviruses. APOIV has, in addition, a C to U change at position 3 of the pentanucleotide motif (table 1) (Charlier, N. *et al.* *J. Gen. Virol.* 2002, 83, 1875-1885). At each end of the genome, two terminal nucleotides, i.e. 5'-AG and CU-3', are conserved among members of the whole *Flavivirus* genus (vector-borne and NKV).

	Strain	Sequence alignment	S	Position
Tick-borne	TBEV 263	CCCAGA[REDACTED]AUAGUCUGACAA -GGA---	1	11,092
	TBEV <sup>NEU</sup>	CCCAGA[REDACTED]GUAGUCUGACAA -GGA---	2	11,092
	TBEV <sup>HYPR</sup>	CCCAGA[REDACTED]AUAGUCUGACAA -GGA---	3	10,786
	TBEV <sup>VAS</sup>	CCCAGA[REDACTED]ACAGUCUGACAA -GGA---	4	10,878
	LGTV	CCUAGA[REDACTED]AUAGUCUGAAAA -GGA---	5	10,894
	POWV	UCCAGG[REDACTED]AUAGCCUGACAA -GGA---	6	10,790
Mosquito-borne	YFV 17D-213	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	7	10,811
	YFV 17DD	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	8	10,811
	YFV neurotropic	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	9	10,811
	YFV viscerotropic	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	10	10,811
	YFV Trinidad	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	11	10,709
	YFV 85-82H	GUGAG-[REDACTED]UUUGCUCUAGAA -UAA---	12	10,811
	DENV-1	UCCAGG[REDACTED]AACGCCAGAAAA UGGAAU-	13	10,689
	DENV-2	UCCAGG[REDACTED]AACGCCAGAAAA UGGAAU-	14	10,677
	DENV-3	UCCAGG[REDACTED]AACGCCAGAAAA UGGAAU-	15	10,650
	DENV-4	UCCAGG[REDACTED]AGCGCCGCAAGA UGGAAU-	16	10,603
	LIV	CCCAGA[REDACTED]AUAGUCUGACAA -GGA---	17	10,822
	WNV	CACGG-[REDACTED]UGCGCC-GACAU AGGUG--	18	10,916
	KUNV	CACGG-[REDACTED]UGCGCC-GACAA UGGUG--	19	11,345
	JEV	ACUAGG[REDACTED]AGCGCCGAAGUA UGUA---	20	10,928
	MVEV	-CAAGC[REDACTED]CAGCGCCGAACAC UGUG---	21	10,966
	CFAV	-CCUCC[REDACTED]CUUAG-GGAGUU UUGA---	22	10,651
NKV	MMLV	--AAGA[REDACTED]AUUGUCUCAUGA -----	23	10,644
	RBV	U---G-[REDACTED]AUUGC---AUGC UGG----	24	NA
	MODV	UCAAG-[REDACTED]AUUG-CUUACUA UGUA---	25	10,552
	APOIV	GUAAUCC[REDACTED]GUUGGAUUAUAAA UUAUCCU	26	NA

Table 1: The conserved pentanucleotide-sequence (5'-CACAG-3') in the last 61 nucleotides of the 3' UTR of mosquito- and tick-borne flaviviruses is a C(C/U)(C/U)AG sequence for NKV flaviviruses. (The sequences correspond to the sequences listed in the sequence listing as sequences 1-26)

5

Since there is no specific treatment for flavivirus infections, and management of patients with the disease is often extremely problematic, much emphasis has been placed on preventive vaccination. Vaccination is currently possible against YFV (live-attenuated), JEV (inactivated and live-attenuated, i.e. JEV-Vax) and TBEV (inactivated). Different vaccine strategies exist like the use of empirically derived and

10 cDNA-derived live attenuated viruses (non-lethal deletion or point mutation),

recombinant subunit vaccines, inactivated virus vaccines and DNA vaccines. Recent introduction of the so-called "infectious clone technology", which is based on a cDNA copy of an RNA virus genome that can be stably propagated in bacterial plasmid vectors and from which RNA can be transcribed that is infectious following  
5 transfection in the appropriate cells, has opened new opportunities in flavivirus vaccine research. The "chimeric" approach for vaccine development is based on the recent observations that the structural genes of one flavivirus can be replaced with homologous genes from another flavivirus (i.e. ChimeriVax vaccines). In this way properties of flaviviruses (such as virulence, the envelope and replication) can be  
10 modified in a chimeric virus by combining genes of different viruses. Several determinants of specific properties of these flaviviruses have already been identified.

However, in the process of developing new vaccines, several factors, such as immunogenicity, virulence, reactivity and neuroinvasiveness, are still problematic  
15 and have to be controlled. For example a vaccine against dengue virus should induce equally high levels of neutralizing antibodies against all four serotypes to prevent the occurrence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Another problem encountered in the vaccination strategy is the prevention of the spread of viruses from a vaccinated person to a non-vaccinated person by a vector  
20 such as mosquitoes.

The major flaviviruses (Dengue, Japanese encephalitis, tick-borne encephalitis, yellow fever, and West Nile viruses) cause substantial morbidity and mortality each year. Due to modern transportation and the relaxation of mosquito-control measures  
25 there has been a substantial increase of disease caused by flaviviruses. Without effective antiviral drugs, vaccination offers the best chance of decreasing the incidence of these diseases, and live virus vaccines are the most promising and cost effective. However, flaviviruses can recombine, which raises the possibility of recombination between a vaccine strain and wild-type virus resulting in a new virus  
30 with potentially undesirable properties (Seligman SJ and Gould EA, Lancet. 2004 Jun 19;363(9426):2073-5).

In view of the importance of treating or preventing of pathologies induced by viruses, there is a need for therapies and/or vaccination strategies with a reduced or no risk for spreading the virus.

## 5 SUMMARY OF THE INVENTION

The present invention relates to the modification of nucleotide sequence involved in the formation of the 3'LSH in the 3'UTR of vector-borne flaviviruses, more particularly to the modification of one or more nucleotides within or 3' adjacent to  
10 the pentanucleotide sequence 5'-CAC(A/C)G-3' in the 3' UTR of viruses in order to reduce the replication of a virus in an organism or a cell, more particularly in a vector, and to reduce or inhibit the transmission of said virus through such a vector. The present invention also relates to a method to reduce the replication of a virus in an organism, more particularly in a vector, comprising modifying the nucleotide  
15 sequence involved in the formation of the 3'LSH in the 3'UTR of vector-borne flaviviruses, more particularly modifying one or more nucleotides within the CAC(A/C)G pentanucleotide motif in the 3' UTR sequence. The invention further relates to a modified virus comprising a modified 3'UTR region, more particularly a modified CAC(A/C)G pentanucleotide motif in the 3' UTR sequence, its use and  
20 pharmaceutical compositions comprising such a virus. The present invention furthermore relates to a vaccine comprising a nucleotide sequence comprising at least the part of the 3' UTR of a flavivirus comprising the conserved pentanucleotide, characterised in that said 3' UTR nucleotide sequence comprises a modification in the sequence involved in formation of the 3'LSH structure, more particularly comprising  
25 a modified CAC(A/C)G sequence or wherein the pentanucleotide is completely deleted. The present invention relates also to the use of the 3' UTR nucleotide sequence of a flavivirus or a part thereof, modified in sequence involved in the formation of the 3' LSH structure, more particularly, modified in the conserved CAC(A/C)G pentanucleotide sequence to reduce the replication of a virus in an  
30 organism, more particularly in a vector in the preparation of a vaccine.

The present invention relates to the reduction of the replication of viruses in a vector. In a particular embodiment the viruses are RNA viruses, yet more in particular they



are member of the family of the Flaviviridae, still more in particular the viruses are selected from the Flavivirus genus. In a more particular embodiment, the viruses are selected from the vector-borne viruses, such as, but not limited to YFV, Dengue virus or WNV.

5

The invention further relates to a modified virus (sequence) comprising a modified 3'UTR, more particularly a modified conserved CAC(A/C)G pentanucleotide motif within the 3' UTR sequence. In a particular embodiment the modified virus is a vector mediated flavivirus such as YFV, DENV and WNV. In another embodiment  
10 the modified virus is a chimeric virus constructed by combining genes of at least two viruses of which at least one is a flavivirus.

The present invention relates also to the use of said modified viruses in a pharmaceutical composition, such as a vaccine or for the preparation of a  
15 pharmaceutical composition, such as a vaccine.

The present invention furthermore relates to a vaccine comprising a nucleotide sequence comprising at least the part of the 3' UTR of a flavivirus comprising the conserved pentanucleotide, characterised in that said 3' UTR nucleotide sequence  
20 comprises a modified CAC(A/C)G sequence. In a particular embodiment, the vaccine comprises a chimeric virus with a modified CAC(A/C)G pentanucleotide sequence. According to the present invention, the chimeric virus thus comprises a 3'UTR of a vector-borne flavivirus which is modified according to the present invention. More particularly, the chimeric virus for use as a vaccine comprises a modified  
25 CAC(A/C)G sequence.

In another embodiment, the vaccine further comprises other viruses, chimeric viruses or parts thereof.

#### DETAILED DESCRIPTION OF THE INVENTION

30

All flaviviruses that cause diseases in humans either belong to the mosquito- or the tick-borne cluster. The third cluster, i.e. the NKV group, consists of viruses for which no arthropod-borne route of transmission has (yet) been demonstrated (Kuno *et al.*,

1998, *J. Virol.* 72, 73-83). The factor(s) in the flavivirus genome that determine whether a flavivirus is able or not to replicate in vector (cells) have until now not been determined.

The present invention is based on the realization that insight into the determinants  
5 that are responsible for vector specificity is important for the development of  
vaccines against flaviviruses. It is observed that the state of the art chimeric vaccine  
viruses based on the YFV (or DENV) backbone and containing the 3' UTR – and  
thus the pentanucleotide motif CACAG of vector-borne flaviviruses – can still  
replicate in insect cells (see our observations described below) and can thus  
10 theoretically be transmitted by mosquitoes from a vaccinated person to a non-  
vaccinated person.

The present invention relates to the determinants of the flaviviral replication and  
transmission and the use of these determinants in the creation of vaccines or in  
15 therapy.

The present invention is thus also based on the observation that the viral envelope  
proteins (prM+E), which are responsible for the initial interaction (i.e. binding and  
fusion) of flaviviruses with the host cell, are only partly responsible for whether a  
20 flavivirus is or is not infectious to a vector. This was established by investigating  
whether the MODV/YFV chimeric virus, which contains the prM and E genes of the  
NKV flavivirus Modoc virus (MODV) in the genome of a mosquito-borne flavivirus  
YFV 17D, was infectious to mosquito cells. Since the MODV/YFV chimeric virus  
contains the envelope proteins of a NKV flavivirus, it was expected that the chimeric  
25 virus would not be infectious to mosquito cells. However, the chimeric MODV/YFV  
replicated as efficiently in mosquito cells as YFV 17D did. This thus proves that the  
envelope proteins of the NKV flaviviruses are not (solely) responsible for the fact  
that NKV viruses do not replicate in mosquito (vector) cells. Based on these  
observations it was concluded that other determinants must be responsible for the fact  
30 that NKV flaviviruses (such as MODV) do not replicate in mosquito cells and,  
conversely, that vector-borne flaviviruses do replicate in mosquito or tick (cells).

In the prior art, a comparative description of the 3' UTR folding of flaviviruses can be found. Strong support was found for the presence of four different RNA regions (designated I, II, III and IV) in the 3' UTR of MMLV, MODV and RBV, but not in the 3' UTR of APOIV (Fig. 5). The latter is assumed not to have a region I equivalent. Hairpins, conserved motifs, single stranded parts, Y-shaped structures and pseudoknots are present in the regions. The very 3' terminus of the 3' UTR folds in a manner typical for all flaviviruses, forming the 3' LSH structure and a small stem-loop (belonging to region IV and probably coaxially stacking with the long 3' terminal hairpin). The 3' LSH, which preserves its shape despite significant differences in sequence, was calculated to fold in the genome of the four NKV flaviviruses with a similar position of the conserved C(C/U)(C/U)AG motif (45-61 nucleotides from the 3' terminus). The present invention demonstrates that one or more modifications in the nucleotides involved in the formation of the 3' LSH, more particularly in the conserved CAC(A/C)G pentanucleotide sequence and/or the stretch of 10 nucleotides 3' thereof, is critical to determining whether or not a virus is capable of replicating in a vector.

The term "vector" as used herein and unless otherwise stated, refers to an organism, which is not a vertebrate, more specifically not a mammal, most particularly not a human, that can carry a virus and transmit it from one organism, particularly a vertebrate, more particularly a mammal, to another. In an aspect of the present invention, a vector is an arthropod, such as but not limited to ticks and mosquitoes.

The term '3' UTR' of a flavivirus as used herein refers to the sequence 3' of the open reading frame, comprising about 400-700 nucleotides. It has been described to contain elements involved in the regulation of essential functions such as translation, replication or encapsidation of the genome (Khromykh *et al.*, 2001, *J. Virol.* 75, 6719-6728; Proutski *et al.*, 1997a, *J. Gen. Virol.* 78, 1543-1549; 1997b, *Nucleic Acids. Res.* 25, 1194-1202). In vector-borne flaviviruses, the 3' UTR comprises a conserved CAC(A/C)G pentanucleotide (sequence) located approximately 45-61 nucleotides from the 3' terminus. A sequence 'corresponding to a functional 3' UTR of a flavivirus' is a sequence which either corresponds to the 3' UTR of a flavivirus or is derived therefrom, while retaining all of the functions normally performed by

the 3'UTR. Thus such a sequence can contain deletions or mutations (other than those described in the present invention) e.g. in non-functional or linker sequences within the 3' UTR.

- 5 The very 3' terminus of the 3' UTR folds in a manner typical for all flaviviruses, forming the 3' long stable hairpin (LSH) structure and a small stem-loop. Thus the term '3'LSH structure' as used herein refers to a secondary structure formed by the 3' terminus of the 3'UTR of flaviviruses. The formation of the 3' LSH structure involves a discrete number of nucleotides, which varies between the different  
10 flaviviruses. For instance, for YFV strain YFV-17D.204, the 3'LSH structure has been demonstrated to involve nucleotides 68-20 upstream of the 3' UTR terminus (Proutski V. et al. *Journal of General Virology* (1997), 78, 1543-1549). Without being limited to theory, it is proposed that the 3'LSH structure ensures specific functions within the 3'UTR (such as e.g. interaction with intracellular proteins).  
15 Modification of one or more nucleotides within the sequence comprised in the 3'LSH will lead to a modification of said functions.

The term 'conserved CAC(A/C)G pentanucleotide (sequence)' as used herein refers to the pentanucleotide sequence 5'-CACAG-3' or 5'-CACCG-3' located  
20 approximately 45-61 nucleotides from the 3' terminus of vector-borne flaviviruses (as described by Wengler & Castle, 1986, *J. Gen. Virol.* 67, 1183-1188; see also Table 1 of introduction). The conserved CAC(A/C)G pentanucleotide is part of the 3'LSH structure of the 3'UTR of such vector-borne flaviviruses.

- 25 A "modified" CAC(A/C)G pentanucleotide sequence as used herein refers to the CAC(A/C)G nucleotide sequence, but modified by (i) changing or mutating nucleotides into other nucleotides or (ii) deleting or inserting nucleotides. The nucleotides that are referred to can be natural or unnatural (synthetic) nucleotides.

- 30 An isolated nucleotide sequence as used herein refers to a DNA or RNA polynucleotide as present outside its natural environment, i.e. as such or as part of a cloning vector or any other recombinant genetic construct. A nucleotide sequence can comprise the complete genome of a virus (or a sequence corresponding thereto) or

parts thereof. Sequences encoding structural genes (such as envelope proteins) are also referred to as structural regions, while sequences encoding non-structural proteins (such as helicases, replicases, etc;) are also referred to as non-structural regions. Non-coding regions can include both the 5' and the 3' UTR.

5

A 'vaccine' as used herein refers to a composition which, upon introduction into a vertebrate, is capable of directly or indirectly generating a protective immune response to one or more viruses in a vertebrate, without inducing all of the disease symptoms associated with infection of the virus or viruses in a vertebrate. A vaccine  
10 can comprise one or more (DNA/RNA) nucleotide sequences encoding an immunogenic protein or peptide and/or one or more immunogenic proteins or peptides. According to the present invention, the vaccine is either a live attenuated virus or a nucleotide encoding a live attenuated virus. In a particular embodiment of the present invention, the vaccine comprises a mutated virus or chimeric virus.

15

An 'immunogenic composition' refers to a composition which directly induces an immune response when injected into a vertebrate. In the context of the present invention an immunogenic composition comprises one or more parts of the (virally encoded) subunits of the envelope of the virus. The envelope of flaviviruses is  
20 derived from the host cell membrane and comprises virally-encoded transmembrane (M) and envelope (E) proteins.

According to a first aspect, the present invention relates to a nucleotide sequence for use in the preparation of a vaccine against one or more flaviviruses. More  
25 particularly, the invention relates to a sequence comprising a functional 3' UTR of a vector-borne flavivirus or a part thereof comprising the conserved pentanucleotide CAC(A/C)G, wherein one or more nucleotides comprised in the 3' LSH in the 3' terminus of the UTR has been modified. According to a specific embodiment, a nucleotide within the conserved pentanucleotide and/or one or more nucleotides  
30 within a sequence of 10 nucleotides 3' of the pentanucleotide is modified. According to the present invention, such a modification ensures that the virus is to a much lesser extent or no longer able to replicate in a vector.

According to another aspect of the invention, modified viruses are provided comprising the modified nucleotide sequence described above, which have a decreased capacity for replicating within a vector, more particularly have a reduced replication within an arthropod, such as, but not limited to a tick or a mosquito. Thus  
5 a vector-borne virus can be modified into a virus which is essentially no longer vector-borne, while maintaining all of its other features, more particularly its envelope proteins, which are critical for eliciting a specific immune response in the context of vaccination. Moreover, according to a particular embodiment of the invention, the replication of the virus in vertebrates is essentially unmodified, which  
10 is of interest for the efficiency of the vaccine.

According to a particular embodiment of the invention the nucleotide sequence encodes a chimeric virus, i.e. a virus which comprises structural and/or non-structural proteins of more than one virus, more particularly more than one flavivirus. According to a more particular embodiment, the nucleotide sequence encodes a  
15 chimeric virus as based on the Chimerivax<sup>TM</sup> technology, whereby a live attenuated recombinant virus is constructed from yellow fever virus (YFV) 17D, or another flavivirus such as dengue virus (DENV) in which the envelope protein genes (prM+E) of the parent genes of YFV17D are replaced by the corresponding genes of another flavivirus (for example, but not limited to JEV, WNV, DENV), as is the case  
20 with the ChimeriVax<sup>TM</sup> vaccines. Further examples of chimeric virus vaccines, more particularly chimeric flavivirus vaccines, are described in the art and include the live, attenuated chimeric virus vaccine against tick-borne encephalitis virus comprising the preM and E structural genes of the tick-borne encephalitis Langat virus and the non-structural genes of the mosquito-borne dengue virus (described in US 6,497,884).  
25 Thus, according to a particular embodiment of the invention, the nucleotide sequence and the virus of the present invention correspond to a chimeric virus comprising at least the 3'UTR of a vector-borne flavivirus, more particularly the non-coding regions of the vector-borne flavivirus, most particularly the non-coding as well as the coding regions of a vector-borne flavivirus, with the exception of one or more genes  
30 encoding structural proteins, whereby one or more nucleotides comprised in the 3'LSH of the UTR, more particularly, one or more nucleotides within the conserved CAC(A/C)G pentanucleotide have been modified. By reducing the ability of the 'backbone' virus (i.e. the virus providing the backbone sequence in which the

structural genes of the virus of interest are introduced) to replicate in a vector, transmission of the virus through a vector is prevented.

The present invention relates thus to a method for reducing the replication of a virus in an organism or a cell, more particularly in a vector or vector-cell, comprising  
5 modifying the 3'UTR, more particularly modifying the sequence which is part of the 3'LSH structure within the 3'UTR, most particularly modifying the conserved CAC(A/C)G sequences therein. Envisaged modifications include those that inhibit hybridisation between complementary nucleotides (A-U/T and C-G) potentially required for the formation of the secondary structure). Most particularly, the  
10 modification involves one or more nucleotides within the conserved CAC(A/C)G pentanucleotide motif in the 3' UTR sequence and/or the sequence of 10 nucleotides 3' thereof, more particularly those present in the 3'LSH structure and responsible for the interaction of the 3'LSH structure with intracellular proteins. The pentanucleotide can be modified in different ways. Nucleotides can be changed or mutated into other  
15 nucleotides, nucleotides can be deleted or nucleotides can be added in the pentanucleotide sequence. In a particular embodiment, the positions 2 and 3 of the pentanucleotide starting from the 5'-end are modified, more in particular are changed or mutated into another nucleotide. Therefore, the pentanucleotide can be modified into C(U/T/G/C)(A/T/G/U)AG, or using synthetic nucleotides. and any combination  
20 thereof. In another particular embodiment, the pentanucleotide is changed or mutated into 5'-CUCAG-3'. Additionally or alternatively, and more particularly for YFV, the modification involves one or more of the U nucleotides 3' of and adjacent to the conserved pentanucleotide. Most particularly, the modification involves the third nucleotide, 3' of the conserved pentanucleotide.

25 Besides the substitutions with naturally occurring nucleotides, modification or replacement by synthetic nucleotides is also envisaged within the context of the present invention. Several modifications of oligonucleotides have been described in order to increase their stability. Examples of such modifications are oligonucleotides containing modified backbones such as phosphorothioate backbones and  
30 oligonucleosides with heteroatom backbones, or non-natural internucleoside linkages. Oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more

short chain heteroatomic or heterocyclic internucleoside linkages. Alternatively, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units can be replaced with novel groups, such as peptide nucleic acids (PNA - Nielsen et al. 1991, Science 254: 1497-1500). Oligonucleotides may also include naturally  
5 occurring or synthetic modifications of the "natural" purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U).

According to the present invention, the 3'UTR of vector-borne flaviviruses is modified, more particularly the natural function of the 3'LSH structure therein is  
10 interfered with, most particularly by modification of the conserved CAC(A/C)G pentanucleotide, so as to reduce replication in a vector. In a particular embodiment, the replication of the virus in vector cells is reduced for 100 % and thereby completely inhibited. The reduction of the replication of the virus in vector cells can however be between 95 and 100%, can be higher than 95%, than 90%, 80%, 70%,  
15 60% or 50%. In a particular embodiment, the replication of the virus in a vector is reduced with at least 20 %. Reduction of replication in cell cultures can be established in different ways, based on the production of viral RNA or protein (such as, but not limited to virus-specific real-time quantitative RT-PCR, as described herein, or virus-specific ELISA) or based on cellular features (such as virus-induced  
20 cytopathic effects). In order to establish reduction of replication a comparison can be made with the virus or nucleotide not modified according to the present invention.

According to a particular embodiment of the present invention, replication of a vector-borne virus within a vector or cells thereof is reduced. The vector in which the replication is reduced can be an arthropod or cells thereof, more in particular an  
25 arthropod known as a vector for certain viruses, such as mosquitoes or ticks.

Additionally, according to a more particular embodiment of the present invention, replication of the vector-borne virus in cells of the host, e.g. the vertebrate, more particularly the mammal is not significantly reduced as a result of the modification of the present invention, thus allowing replication of the virus in a  
30 vertebrate. This is of interest, e.g., for the propagation of the vaccine within the vaccinated host. Optimally, according to the present invention, replication of at least 5-10%, more particularly at least 10-20%, most particularly at least 20-50% is retained in vertebrate, more particularly mammal cells.



The nucleotides sequences and viruses of the invention modified in the 3' UTR, more particularly modified in one or more nucleotides present in the 3'LSH structure therein, most particularly modified in one or more nucleotides within the conserved pentanucleotide sequence CAC(A/C)G can be used for the preparation of a pharmaceutical composition, like a vaccine or can be used in a vaccine.

In order for the virus or a part thereof to be used as a vaccine, further modifications may be required to ensure its suitability as a vaccine, i.e. sufficient immunogenicity, while not inducing all physical symptoms of the disease normally associated with the introduction of the virus into the body. More particularly, for YFV or JEV it is desirable that the attenuated virus (as such or as produced in vivo upon vaccination with a nucleotide sequence) is less (not) neuroinvasive and less (not) neuropathogenic.

Methods for reducing the pathogenicity of a virus, resulting in an 'attenuated' virus are known to the skilled person and include but are not limited to mutations in the coding and non-coding regions.

In a naturally attenuated tick-borne flavivirus, Langat (LGT) strain TP21, recovered from ticks in Malaysia, increase in attenuation was associated with three amino acid substitutions, two located in the structural protein E and one in nonstructural protein NS4B (Pletnev AG, Virology. 2001 Apr 10;282(2):288-300). The Dengue virus type 4, attenuated versions were obtained by deletions in 5' non-coding (NC) region (i.e., 5'd (82-87), 5'd (73-77), and 5'd (76-81)). Introduction of mutations into the non-structural genes or 3' untranslated region of an attenuated dengue virus type 4 was found to further decrease replication in rhesus monkeys while retaining protective immunity (Hanley KA et al., Vaccine. 2004 Sep 3;22(25-26):3440-8). rDENdelta30 is a dengue virus vaccine candidate which comprises a 30 nucleotide deletion in the 3' untranslated region about 100 nucleotides upstream of the 3'LSH.

Alternatively, a non-infectious RNA vaccine has been described based on genetic modifications in the region encoding the capsid protein which simultaneously prevents the assembly of infectious virus particles and promotes the secretion of noninfectious subviral particles that elicit neutralizing antibodies (Kofler RM, Proc Natl Acad Sci U S A. 2004 Feb 17;101(7):1951-6. Epub 2004).

Alternatively, a chimeric virus is provided, i.e. a combination of e.g. the structural genes of the virus of interest (against which a protective immune response is desired) and the non-structural genes of another virus). The ChimeriVax<sup>TM</sup> system approach replaces the E gene of the 17D yellow fever vaccine with the analogous  
5 gene of the vaccine-targeted flavivirus, and has been used to obtain JE, DEN and West Nile vaccines. Such chimeric viruses have been demonstrated to show lower neurovirulence than the parent virus while inducing a dose-dependent virus-neutralizing antibody response (Lai CJ, Monath TP, Adv Virus Res. 2003;61:469-509).

10 Antigenic chimeric viruses in which the structural genes (the capsid, membrane precursor, and envelope (CME) or the membrane precursor and envelope (ME) gene regions) of dengue virus type 4 (DEN4) have been replaced with the corresponding genes of dengue virus type 2 (DEN2) have been created to obtain a live attenuated tetravalent dengue virus vaccine (Whitehead SS, Vaccine. 2003 Oct  
15 1;21(27-30):4307-16). In a particular embodiment of the present invention, the modification of the 3'UTR, more particularly the modification of the pentanucleotide and/or one or more of the nucleotides 3' thereof is combined with modifying the envelope or a certain part of the envelope of said virus into the envelope of a NKV.

20 The modified virus can at least be mixed with a pharmaceutically acceptable carrier. Suitable pharmaceutical carriers for this purpose are described for instance in Remington's Pharmaceutical Sciences 16<sup>th</sup> ed. (1980) and their formulation is well known to those skilled in the art. They include any and all conventional solvents, dispersion media, coatings, antibacterial and antifungal agents (for example phenol,  
25 sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like. Additional ingredients may be included in order to control the duration of action of the active ingredient in the composition.

Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in  
30 saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such

as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers.

The "pharmaceutical composition" may be administered by any suitable method within the knowledge of the skilled man. To immunize subjects against  
5 flaviviral infection, the vaccines containing immunologically effective amounts of the virus are administered to the subject in conventional immunization protocols involving, usually, multiple administrations of the vaccine. Administration is typically by injection, typically intramuscular or subcutaneous injection; however, other systemic modes of administration may also be employed. Less frequently used,  
10 transmucosal and transdermal formulations are included within the scope of the invention as are effective means of oral administration. The efficacy of these formulations is a function of the development of formulation technology rather than the contribution of the present invention. The preferred route of administration is parenterally. In parental administration, the composition of this invention will be  
15 formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. In a particular embodiment, it is given as a bolus dose.

20 The following examples, not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1. Genomic organization of the *Flaviviruses*.

25 Figure 2. Growth kinetics of YFV (□), MODV/YFV (●) and MODV (▲) in Vero and mosquito cells. Quantification of viral RNA in the supernatant was performed in triplicate by real-time quantitative RT-PCR using the probe methodology (standard deviation of triplicate determination below 15%) (PCRU = PCR units: One PCR unit is defined to be the lowest template  
30 copy number detectable in three of three replicative reactions as determined by limiting dilution series).

Figure 3. Growth kinetics of MODV/YFV(CACAG) (●) and the mutant chimeric virus MODV/YFV(CUCAG) (○) in Vero and mosquito cells. Two independent experiments were carried out that yielded similar findings. Data from one experiment are shown. Quantification of viral RNA in the supernatant was performed in duplicate by real-time quantitative RT-PCR using the probe methodology (standard deviation of triplicate determination below 15%).

Figure 4. Construction of MODV/YFV chimera by using a variant of the fusion-PCR

Figure 5. Proposed secondary structure of the 3' UTR of four NKV flaviviruses. The four regions (labelled I to IV) are delineated by boxes. Conserved motifs are shown in bold and boxed. For MMLV and RBV, the predicted pseudoknot is shown by connecting boxes. For MMLV and MODV, possible stem-loops are connected by dotted lines.

Figure 6. Growth kinetics of MODV/YFV(CACAG) (--♦--), of wild-type YF (--■--), and the mutant chimeric viruses MODV/YFV(CTCAG) (—▲—), MODV/YFV(CCCAG) (—■—) and MODV/YFV(CGCAG) (—●—) in Vero and mosquito cells ('mug').

Figure 7. Growth kinetics of MODV/YFV(CACAG) (--♦--), of wild-type YF(--■--) and the mutant chimeric virus MODV/YFV(CTCAG) (—▲—) in Vero and mosquito cells ('mug').

## Examples

### Example 1: Materials and methods

Unless otherwise indicated, all the buffers used for restriction enzymes, ligases, and polymerases were provided by the suppliers and used according to their specifications.

*Cells, viruses and plasmids*

Vero and BHK-21 cells were originally obtained from the ATCC (CCL-81 and CCL-10 respectively). The clone pACNR-MODV/YFV was constructed as described in hereunder (Charlier et al., 2003, *J. Virol. Methods* 108, 67-74; ). Mosquito (C6/36, *Aedes albopictus*) cells and the clone pACNR-FLYF17Da can be obtained from the researchers.

*Amplification and cloning of a short YFV fragment containing the CACAG pentanucleotide sequence*

A fragment of 590 bp (nt-position 10,694-11,283 of the YFV 17D genome), containing the CACAG pentanucleotide motif, was amplified in a reaction mixture of 50 µl consisting of 30 ng of pACNR-FLYF17Da plasmid, 2 units of *Pfu* DNA polymerase (Promega), 5 µl of 10 x buffer supplied by the Manufacturer, 400 µM dNTP, and 1.2 µM of each of the two primers (sense primer 5'-GTAGAAAGACGGGGTCTAGAGGT-3' (SEQ ID NO:27) and antisense primer 5'-GGCACTGATGAGGGTGTCTAGTG-3' (SEQ ID NO: 28). The conditions for the amplification reaction were as follows: 30 s at 95°C, 30 s at 60°C and 1 min at 72°C repeated for 25 cycles.

Following agarose gel electrophoresis the DNA fragment with the expected length was cloned into a TOPO vector using the TOPO TA Cloning kit (version H) (Invitrogen) and One Shot TOP10 *E. coli* cells (Invitrogen) to yield pYFV-CACAG.

*Mutation of the pentanucleotide sequence*

The adenosine at position two of the pentanucleotide motif CACAG was mutated into a thymidine using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Texas, USA): 50 ng of the pYFV-CACAG plasmide was added to 5 µl 10 x buffer supplied by the Manufacturer, 400 µM dNTP, 0.22 µM of each of the two primers (sense primer and antisense primer 5'-GAGGTCTGTGAGCTCAGTTTGCTCAAGAATAAGCAG-3' (SEQ ID NO: 29) and 2.5 units of *Pfu* Turbo DNA polymerase. The conditions for the amplification reactions were 30 s at 95°C, 1 min at 55°C and 9 min at 68°C repeated for 12 cycles. Following amplification, the sample was treated with 10 units of the *DpnI* restriction enzyme and transformed in One Shot TOP10 *E. coli* cells (Invitrogen) to yield pYFV-CTCAG. To assure that the

pentanucleotide sequence was altered correctly and that no other mutations were inserted into the genome, the exchanged fragment was sequenced.

*Construction of the MODV/YFV full-length clone containing the CUCAG motif*

- 5 The plasmids pYFV-CTCAG and pACNR-MODV/YFV were digested with the restriction enzymes *XbaI* and *HaeII* (Promega), and the CTCAG containing fragment was inserted in the full-length clone pACNR-MODV/YFV using T4 DNA ligase (Promega). Following ligation, which yielded the plasmid pACNR-MODV/YFV(CTCAG), the latter was transformed into MC1061 *E. coli* cells.

10

*RNA transcription and transfection*

- Recombinant viral RNA was transcribed from 5 µg of *AflII*-linearized pACNR-MODV/YFV(CTCAG) using Sp6 RNA-polymerase (mMessage mMachine Kit; Ambion Ltd., Cambridgeshire, United Kingdom). BHK cells were transfected by  
15 electroporation as described (van Dinten *et al.*, 1997, Proc Natl Acad Sci U S A. 1997 Feb 4;94(3):991-6). Cell culture medium was harvested at the time that the transfected cells displayed nearly complete CPE. Medium was cleared from cell debris by centrifugation and subsequently used to prepare layer stocks in Vero cells.

20 *Monitoring viral kinetics*

- Monolayers of Vero and C6/36 cells were inoculated with  $10^7$  pfu of either MODV, YFV, MODV/YFV(CACAG) or the MODV/YFV(CUCAG) chimeric virus at 37°C and 28°C in 25 cm<sup>2</sup> culture flasks. Cell culture medium was harvested every day or every two days (between day 0 and 10), and titrated for infectious virus content on  
25 Vero cells. Viral RNA load was determined by real-time quantitative RT-PCR (see below) on RNA extracted from the collected media.

*Quantitative RT-PCR of MODV, YFV, MODV/YFV(CACAG) and MODV/YFV(CUCAG) RNA*

- 30 RNA extraction was performed using the Qiagen Viral RNA kit (Qiagen) according to the Manufacturer's instructions. For elution of RNA, the columns were incubated with 50 µl of RNase-free water at 80 °C. For the quantitative determination of MODV, YFV and MODV/YFV RNA in the supernatant of infected Vero and C6/36

cells, the reaction conditions were as following. Primers and probes were designed for MODV/YFV 17D: sense primer 5'-TGGGTTTTGGTCTTCTAGCTTTCA-3' (SEQ ID NO:30), antisense primer 5'-CTTGTTTCAGCCAGTCATCAGAGTCT-3' (SEQ ID NO: 31) and probe 5'-CAGGAGTGATGGGAAATCAAGGATGC-3' (SEQ ID NO: 32); YFV 17D: sense primer 5'-AATCGAGTTGCTAGGCAATAAACAC-3' (SEQ ID NO: 33), antisense primer 5'-TCCCTGAGCTTTACGACCAGA-3' (SEQ ID NO: 34) and probe 5'-ATCGTTCGTTGAGCGATTAGCAG-3' (SEQ ID NO: 35). Primers and probe for quantitation of MODV RNA and the reaction conditions for MODV/YFV 17D, YFV 17D and MODV were as reported earlier (Leyssen *et al.*, 2001, *Virology* 279, 27-37). Thermal cycling in a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) involved reverse transcription (RT) at 45°C for 20 min, denaturation at 95°C for 5 min, followed by 45 cycles of 5 s at 95°C and 35 s at 57°C (for YFV) or 20 s at 60°C (for MODV and MODV/YFV) (Wittwer *et al.*, 1997, *Biotechniques* 22, 176-181; Drosten *et al.*, 2002, *J. Clin. Microbiol.* 40, 2323-2330).

*Rapid and efficient method to create chimeric viruses (i.e. Construction of the clone pACNR-MODV/YFV)*

Cells, viruses and plasmids: Vero and BHK-21 (Baby Hamster Kidney) cells, and MODV were originally obtained from the ATCC (CCL-81, CCL-10 and VR-415 respectively). MODV was grown in Vero cells (Leyssen *et al.*, 2001, *Virology* 279, 27-37). The clone pACNR-FLYF17Da contains a full-length cDNA of YFV 17D and is identical to pACNR-FLYF17Dx (Bredenbeek *et al.*, 2003, *J. Gen. Virol.* 84, 1261-1268) except for the *XhoI* transcription run-off site which was changed to an *AflIII* site in pACNR-FLYF17Da. Plasmid pHYF-5' is a derivative of pHYF-5'3'IV (Bredenbeek *et al.*, 2003, above) and contains a *NotI-MluI* fragment encompassing the Sp6 promoter fused directly to the 5' 2947 bases of YFV 17D.

Amplification and cloning of MODV prM+E cDNA: MODV RNA was extracted from 140 µl of cell culture supernatant using the QIAamp Viral RNA kit (Qiagen) according to the Manufacturer's instructions. The cDNA was synthesized and amplified using the One Step RT-PCR kit (Qiagen): 5 µl MODV RNA was added to 10 µl 5 x RT-PCR buffer, 0.4 mM dNTP, 2 µl enzyme-mix, 95 units of HPRI (Amersham Pharmacia Biotech), 0.6 µM of each primer [sense primer, 5'-

AAGGTTTTGGAAGATGACTCCGGC-3' (SEQ ID NO: 36) (nt-position 271-294); antisense primer, 5'-GTTAATGACTGGTATGGGGGGTACA-3' (SEQ ID NO: 37)(nt-position 2444-2468)] and 29 µl RNase-free water in a final volume of 50 µl. The following amplification program was used: an RT at 50°C for 30 min, an initial PCR activation step of 15 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 72°C and a final extension phase of 10 min at 72°C. The DNA fragment with the expected length of 2.2 kb was cloned into a TOPO vector using the TOPO TA Cloning kit (version H) (Invitrogen) and One Shot TOP10 *E. coli* cells (Invitrogen) to yield pMODV-prM+E.

**Primers:** Primers used for the construction of the chimeric region were designed based on the nucleotide sequence of YFV 17D (GenBank accession number X03700) (Rice *et al.*, 1985 *Science* 229, 726-733) and MODV (GenBank accession number NC\_003635) (Leyssen *et al.*, 2002, *Virology* 293, 125-140). The nucleotide sequences of the primers are listed in Table 2.

Table 2

Primers used for fusion-PCR

Name	Polarity	Sequence	
A	S	5'-GGAATGCTGTTGATGACGGGTGGAACCATATTGTCAATTGAAGTTGTT-3' (38)	Y/M
B	AS	5'-GCACTGAGGATCCCAATGGAA-3' (39)	M
C	S	5'-GCAACGCGCGCGCGCTAGCGATGACC-3' (40)	Y (Not)
D	S	5'-CTTTCACCACAGGAGTGATGGGAGATCAAGGATGCGCCATCAACTT-3' (41)	M/Y
E	AS	5'-GCCTAAATTCAGTTGACTCC-3' (42)	Y
F	S	5'-TGCCTGTTGAAAAGGATCGT-3' (43)	M

Abbreviations: S: sense; AS: antisense; Y: YFV; M: MODV. (Numbers between brackets refer to sequence listing)

**Fusion-PCR:** Two short fragments (205 bp and 209 bp, Fig. 4), that were to serve as primers in the subsequent fusion-PCR, were amplified in a reaction mix of 50 µl consisting of 100 ng (pHYF-5' or pMODV-prM+E) plasmid, 2 units of *Pfu* DNA polymerase (Promega), 5 µl of 10 x buffer supplied by the Manufacturer, 400 µM dNTP, and 1.2 µM of each of the two primers (Table 2, A+B and D+E respectively). The conditions for the amplification reactions were as follows: 30 s at 95°C, 30 s at 50°C and 1 min at 72°C repeated for 25 cycles.



For the fusion-PCR, the reaction mix of 50 µl consisted of 50 ng (pHYF-5' or pMODV-prM+E) plasmid, 2 units of *Pfu* DNA polymerase (Promega), 5 µl of 10 x buffer supplied by the Manufacturer, 400 µM dNTP, and 1.2 µM of each of the two primers (Table 2, C and F respectively). The conditions for the amplification reactions were as follows: 1 min at 95°C, 1 min at 59°C and 2 min at 72°C repeated for 35 cycles. Two fragments of 933 bp and 2051 bp respectively were thus obtained (Fig. 4).

Construction of the recombinant plasmid: The fragment of 2051 bp and the pHYF-5' vector were digested with *SacI* and *HpaI* (Promega) (Fig. 4). Ligation of 100 ng vector with 65 ng PCR fragment was carried out using the T4 DNA ligase in a 2 x rapid ligation buffer (New England Biolabs GmbH, Frankfurt am Mainz, Germany). The resulting plasmid pHYF-MO1 was digested with *HpaI* and *NotI* (Promega) and served as a vector for the insertion of the *HpaI-NotI* digested 933 bp fragment. The resulting plasmid pHYF-MO2 was digested with *MluI-NotI* (Promega) and the DNA fragment [encompassing from 5' to 3' the Sp6 promotor fused to the YFV 5' UTR and C gene, MODV prM and E gene and part of the YFV NS1 gene] was ligated into *NotI-MluI* digested pACNR-FLYF17Da to construct pACNR-MODV/YFV (Fig. 4). The recombinant region was sequenced in a cycle sequencing reaction with fluorescent dye terminators (Big Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems Division) and analyzed using an ABI 373 automatic sequencer (Applied Biosystems Division).

Generation of recombinant viral RNA and transfection of BHK cells: Recombinant viral RNA was transcribed *in vitro* for 2 h at 37°C using 5 µg *AflIII*-linearized pACNR-MODV/YFV plasmid (Fig. 4) as a template and Sp6 RNA-polymerase (1500 units/ml) and RNase inhibitor (1000 units/ml) using the reaction conditions provided by the supplier. The transcription reaction was spiked with 1 µCi [<sup>3</sup>H]-UTP (46 Ci/mmol) to determine the yield of the transcription reaction.

BHK cells were transfected by electroporation essentially as described (van Dinten *et al.*, 1997, Proc Natl Acad Sci U S A. 1997 Feb 4;94(3):991-6). The medium was harvested from the transfected cells when the CPE was nearly complete, clarified by centrifugation and subsequently used to infect new Vero cells. At 48 h post infection total cellular RNA was isolated from the infected Vero cells and subsequently used in a RT-PCR to verify the chimeric nature of the virus.

Example 2: Replication kinetics of MODV, YFV and MODV/YFV in Vero and mosquito cells

To determine whether prM and E of flaviviruses play a role in the vector specificity, the replication efficacy of MODV, YFV and MODV/YFV was compared in Vero and mosquito cells. The kinetics of replication of the three viruses were assessed by means of virus specific real-time quantitative RT-PCR. All viruses reached a plateau of virus production in Vero cells approximately 6 days post infection (Fig. 2). YFV 17D grew as efficiently in mosquito C6/36 cells as in Vero cells. As expected, the NKV flavivirus MODV did not replicate in C6/36 cells. If one assumes that the prM and E proteins of MODV are responsible for the fact the virus does not replicate in mosquito cells, one would expect the chimeric virus MODV/YFV (containing the prM and E of MODV) not to replicate in mosquito cells. However, in contrast to expectations, the MODV/YFV replicated as efficiently as the parental YFV 17D in mosquito C6/36 cells (Fig. 2). The factors that determine whether a flavivirus is able to replicate in mosquito cells, are thus not located in the envelope proteins prM and E.

Example 3: Replication kinetics of MODV/YFV(CACAG) and MODV/YFV (CUCAG) in Vero and mosquito cells

The pentanucleotide sequence CACAG in the 3' LSH of the 3' UTR in MODV/YFV was mutated into the pentanucleotide motif that is characteristic for NKV flaviviruses. The course of replication of the parental MODV/YFV(CACAG) and mutant chimeric MODV/YFV(CUCAG) in Vero and C6/36 cells was compared in two independent experiments. The kinetics of replication of the two viruses was assessed by means of real-time quantitative RT-PCR. In Vero cells, the chimeric MODV/YFV that contains the NKV pentanucleotide motif, replicated somewhat slower than the chimeric virus that contains the vector-borne CACAG motif. Eventually, both viruses efficiently produced CPE in these cells. In contrast, the chimeric MODV/YFV virus that contained the NKV specific pentanucleotide motif CUCAG, was not longer able to replicate in mosquito (C6/36) cells (Fig. 3).

To study whether the inability of MODV/YFV(CUCAG) to replicate in mosquito cells was solely due to the mutation in the pentanucleotide motif (CACAG →

CUCAG), the entire genome of the virus was sequenced. No additional mutation was found.

5 Example 4: Replication kinetics of MODV/YFV(CACAG) and MODV/YFV  
10 (C(C/G)CAG) in Vero and mosquito cells

Monkey kidney cells (Vero cells) and mosquito cells (C6/36) were infected for two hours (day 0) with a) yellow fever virus, b) the original MOD/YF chimeric virus (with CACAG motif) and mutants thereof having c) the motif CTCAG, d) the motif  
10 CCCAG and e) the motif CGCAG, according to the procedures described above.

In a first experiment, samples were assayed on day 0, 3 and 8 (Figure 6). In a second experiment, samples were assayed daily during a period of 8 days (Figure 7).

All viruses replicated well in the mammalian monkey kidney cells. In mosquito cells however, no replication occurs with the mutant having the CTCAG motif.  
15 [quantitative PCR (Taqman) indicates that replication does occur with the mutants having the CCCAG and the CGCAG motif.]

## CLAIMS

1. An isolated nucleotide sequence comprising a sequence corresponding to a functional 3' UTR of a vector-borne flavivirus or a part thereof comprising the conserved pentanucleotide CAC(A/C)G, characterised in one or more nucleotides present in the 3'LSH within the 3'UTR is modified.
2. The isolated nucleotide sequence according to claim 1, characterized in that one or more nucleotides within said conserved pentanucleotide and/or 10 nucleotides 3' thereof is modified.
3. The isolated nucleotide sequence according to claim 1 or 2, characterized in that one or more nucleotides within said conserved CAC(A/C)G pentanucleotide is modified.
4. The isolated nucleotide of any one of claims 1 to 3, wherein the pentanucleotide is modified into C(U/T/G/C)(A/T/G/U)AG or synthetically modified versions of said nucleotides and any combination thereof.
5. The isolated nucleotide sequence according to any one of claims 1 to 4, which is an RNA corresponding to the complete genome of a flavivirus or a DNA corresponding thereto.
6. The isolated nucleotide sequence according to any one of claims 1 to 5, comprising a non-structural region or a portion thereof of a flavivirus, and a structural region or a portion thereof from a different flavivirus.
7. The isolated nucleotide sequence according to any one of claims 1 to 6, wherein said functional 3'UTR corresponds to a functional 3' UTR of a YFV, DENV or JEV.
8. The isolated nucleotide sequence of any one of claims 1 to 7, wherein one or more structural regions within the coding region have been replaced by one or more structural regions of another virus.
9. A vaccine comprising the nucleotide sequence of any one of claims 1 to 8.
10. A vaccine comprising a nucleotide sequence, at least comprising a functional 3' UTR of a vector-borne flavivirus or a part thereof comprising the conserved pentanucleotide CAC(A/C)G, characterised in one or more nucleotides present in the 3'LSH within the 3'UTR is modified.

11. The vaccine of claim 9 or 10, wherein said one or more nucleotides within said conserved pentanucleotide is modified.
12. The vaccine of any one of claims 9 to 11, wherein said vaccine is a chimeric virus vaccine.
- 5 13. The vaccine of any one of claims 9 to 12, further comprising one or more viruses or parts thereof.
14. The vaccine according to claim 12, comprising one or more non-structural protein or a portion thereof of a flavivirus, and one or more structural proteins or a portion thereof from a different flavivirus.
- 10 15. A method to reduce the replication of a vector-borne flavivirus in an vector comprising modifying one or more nucleotides in the sequence comprised within the 3'LSH structure in the 3'UTR.
16. The method according to claim 15 to reduce the replication of a virus in a vector comprising modifying the conserved CAC(A/C)G pentanucleotide motif in the 3'
- 15 UTR sequence.
17. The method of claim 15 or 16, wherein the replication is not significantly reduced in cells of the vertebrate host of said virus.
18. The method of claims 15 to 17, wherein the pentanucleotide motif is modified in the second and third position from the 5'-side on.
- 20 19. The method of claims 15 to 18, wherein the pentanucleotide is modified into C(U/T/G/C)(A/T/G/U)AG, or synthetic versions of said nucleotides and any combination thereof.
20. The method of claims 15 to 19, wherein the reduction of replication in a vector is higher than 95%.
- 25 21. A modified virus comprising a 3'UTR which has been modified in one or more nucleotides involved in the formation of the 3'LSH structure.
22. The modified virus according to claim 21, which comprises a modified CAC(A/C)G pentanucleotide sequence in the 3' UTR sequence.
23. The modified virus of claim 21 or 22 wherein said modified virus is a chimeric
- 30 virus.
24. The modified virus comprising the nucleotide of any one of claims 1 to 8.
25. The use of the modified virus of any one of claims 21-24 in a vaccine.

26. A pharmaceutical composition comprising the modified virus of any one of claims 21-24.
27. The pharmaceutical composition of claim 26, further comprising one or more viruses or parts thereof.
- 5 28. The use of the 3' UTR nucleotide sequence of a vector-borne flavivirus or a part thereof, modified in the CAC(A/C)G pentanucleotide sequence to reduce the replication of a virus in an organism.
29. The use of the 3' UTR nucleotide sequence of a flavivirus or a part thereof, modified in the CAC(A/C)G pentanucleotide sequence in the preparation of a vaccine.
- 10

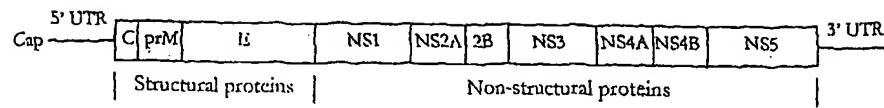


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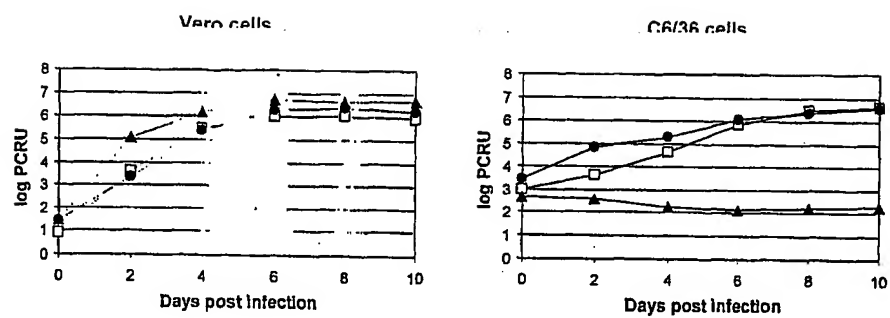


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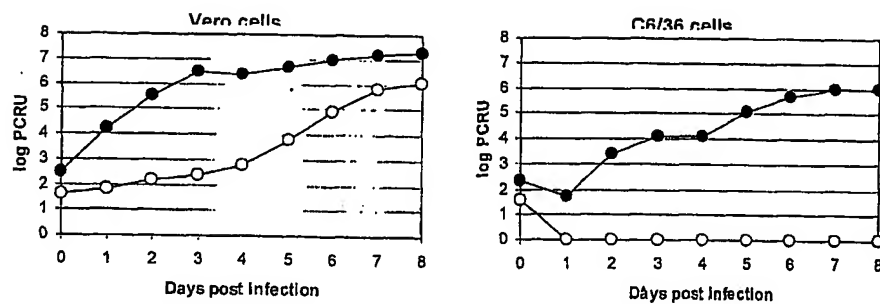


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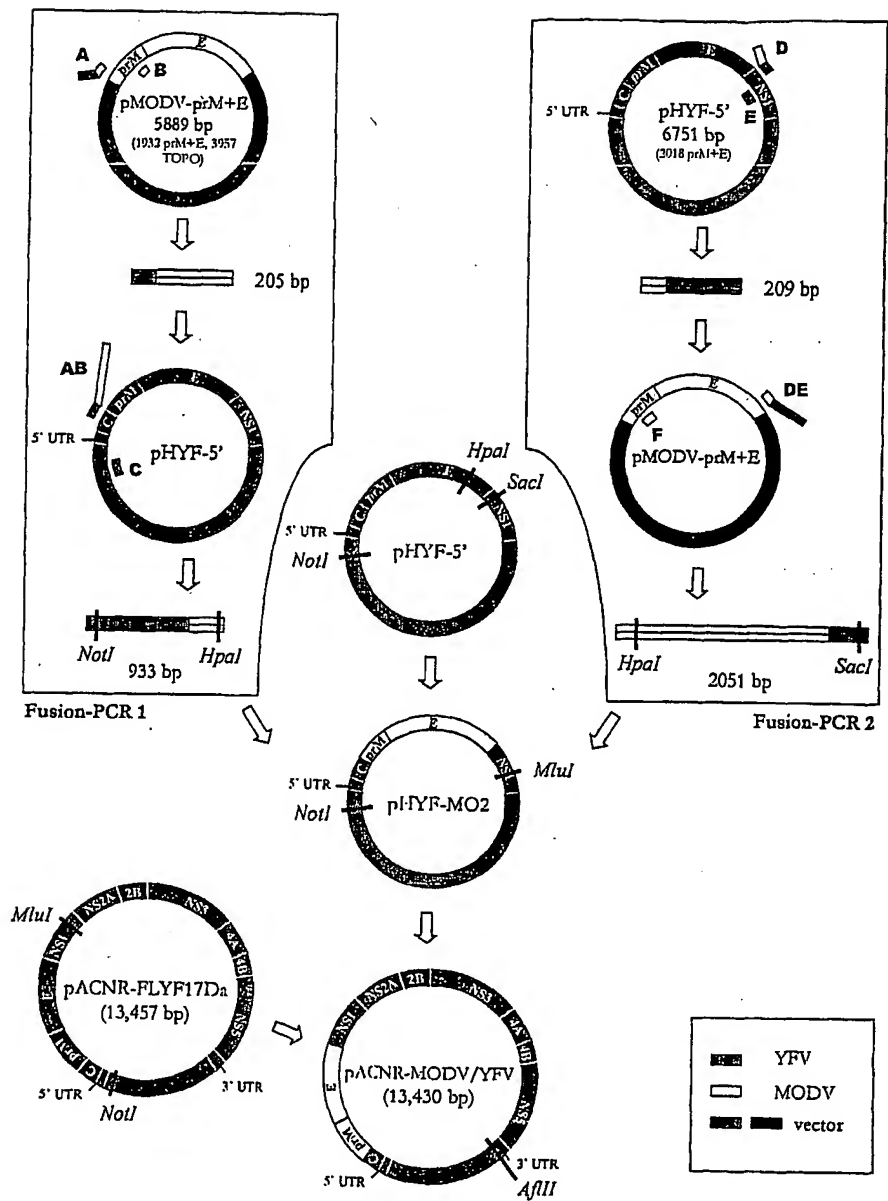


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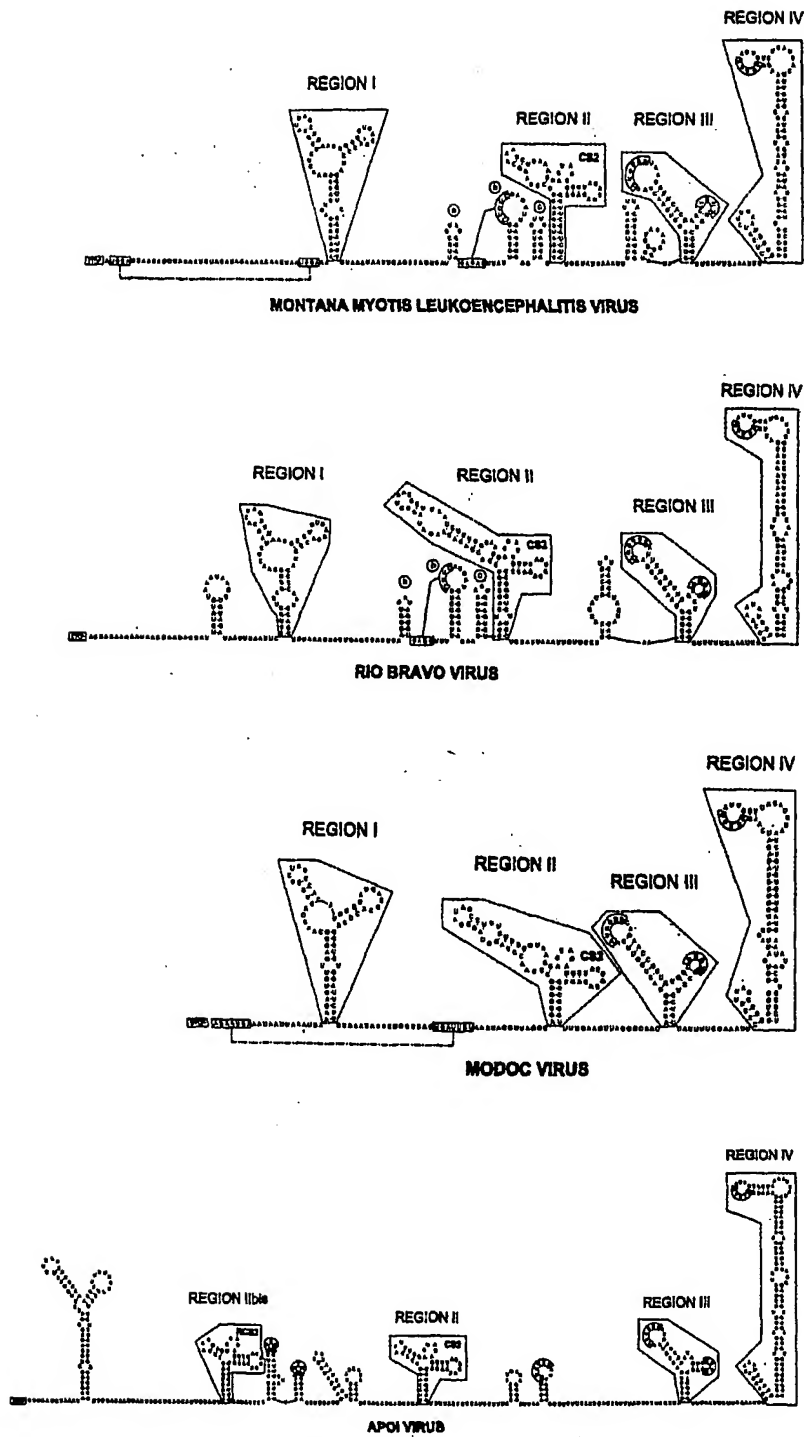


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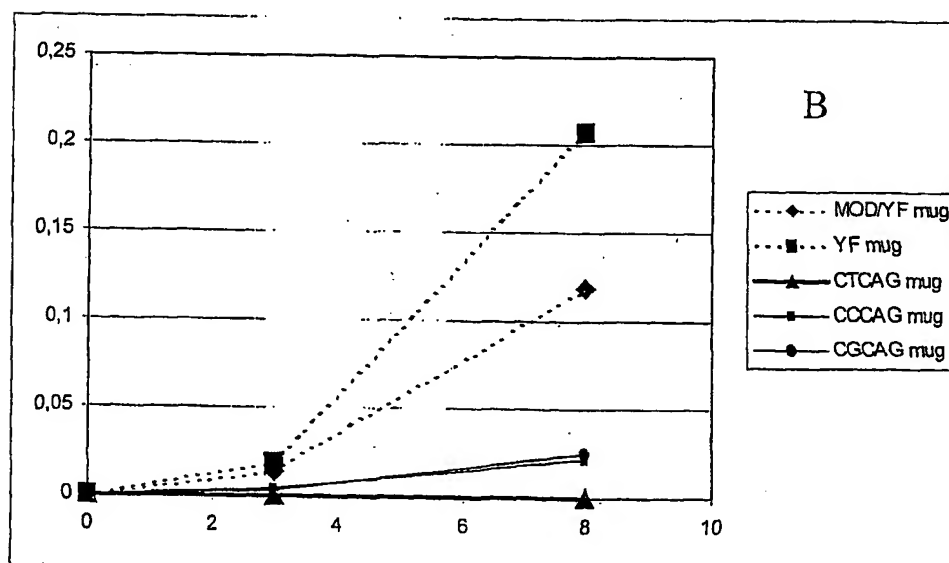
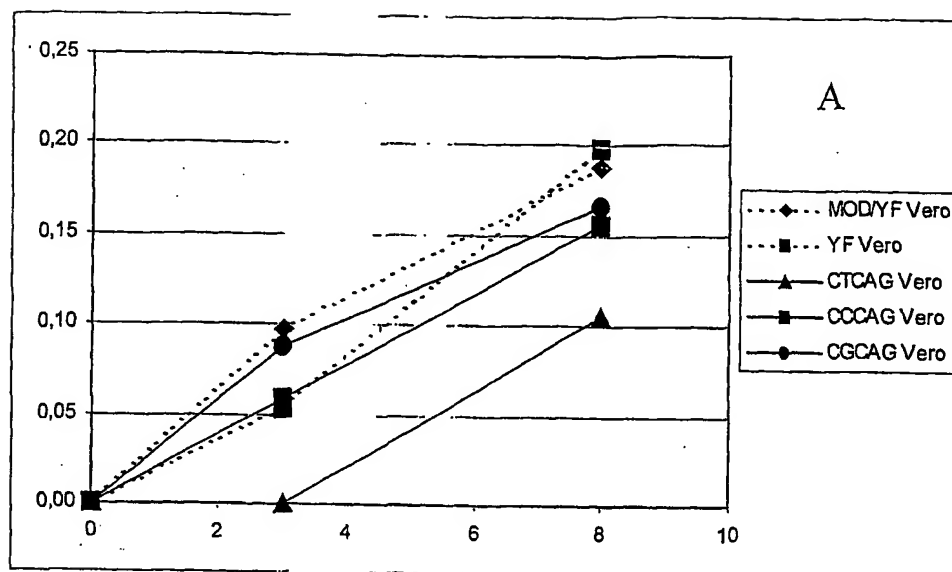


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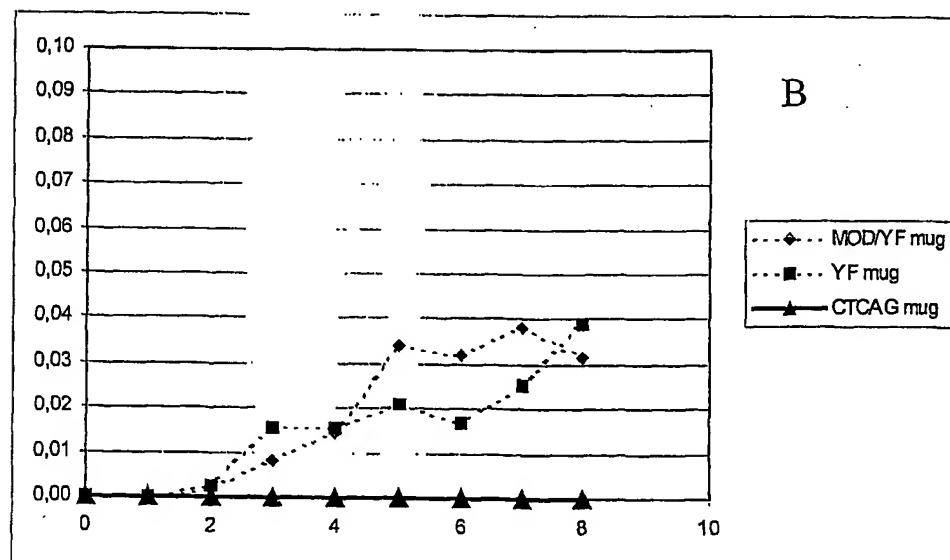
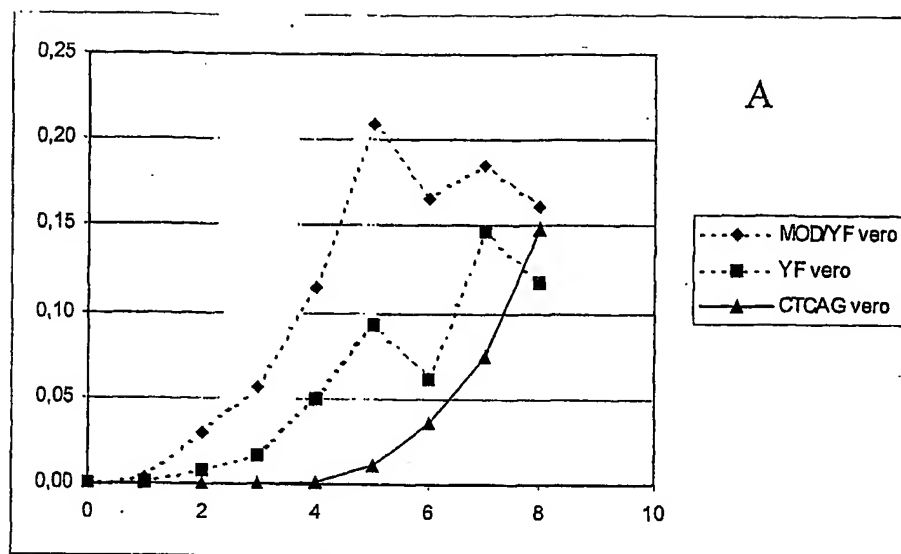


Figure 7

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/BE2004/000166

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N7/04 A61K31/7105 A61K31/711 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X  Y	<p>WO 02/074963 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE S) 26 September 2002 (2002-09-26)</p> <p>page 6, paragraph 3 - page 9, paragraph 3 examples 1,5,6,7,9,10 figures 1-3,5,9,12 claims 1-5,7,8,10-14,16,17,19</p> <p>----- -/-</p>	<p>1,5,7,9, 10,15, 17,20, 21,24-26 6,8, 12-14, 23,27</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the International search

14 April 2005

Date of mailing of the international search report

22/04/2005

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Authorized officer

Tudor, M

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/BE2004/000166

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARKOFF LEWIS ET AL: "Derivation and characterization of a dengue type 1 host range-restricted mutant virus that is attenuated and highly immunogenic in monkeys." JOURNAL OF VIROLOGY. APR 2002, vol. 76, no. 7, April 2002 (2002-04), pages 3318-3328, XP002324288 ISSN: 0022-538X	1,5,7,9, 10,15, 17,20, 21,24-26
Y	abstract page 3318, right-hand column, line 11 - page 3319, left-hand column, line 6 page 3323, right-hand column, lines 34-38 page 3325, left-hand column, paragraph 2 page 3327, right-hand column, paragraph 3 - page 3328, left-hand column, paragraph 1	6,8, 12-14, 23,27
X	BLACKWELL JERRY L ET AL: "Translation elongation factor-1 alpha interacts with the 3' stem-loop region of West Nile virus genomic RNA" JOURNAL OF VIROLOGY, vol. 71, no. 9, 1997, pages 6433-6444, XP002324290 ISSN: 0022-538X page 6439, right-hand column, lines 27-30 page 6440, right-hand column, lines 8-11 page 6442, right-hand column, lines 33-36 RNA 5 of figure 8	1-3
X	KHROMYKH ALEXANDER A ET AL: "Significance in replication of the terminal nucleotides of the flavivirus genome." JOURNAL OF VIROLOGY. OCT 2003, vol. 77, no. 19, October 2003 (2003-10), pages 10623-10629, XP002324289 ISSN: 0022-538X	1-3,5, 15,16,18
Y	page 10627, left-hand column, paragraph 2 - right-hand column, paragraph 1  -/--	4,11,19, 22,28,29

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/BE2004/000166

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>CHARLIER NATHALIE ET AL: "Complete genome sequence of Montana Myotis leukoencephalitis virus, phylogenetic analysis and comparative study of the 3' untranslated region of flaviviruses with no known vector."  THE JOURNAL OF GENERAL VIROLOGY. AUG 2002, vol. 83, no. Pt 8, August 2002 (2002-08), pages 1875-1885, XP002324544  ISSN: 0022-1317  table 4  page 1880, left-hand column, line 2 - page 1881, left-hand column, line 3  page 1884, left-hand column, paragraph 4 - right-hand column, paragraph 1</p>	<p>4,11,19, 22,28,29</p>

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/BE2004/000166

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02074963 A	26-09-2002	WO 02074963 A1	26-09-2002